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FUNCTIONAL CONSEQUENCES OF CHEMICAL MODIFICATION OF THE SAXITOXIN BINDING SITE  
ON NEURONAL SODIUM CHANNELS.

Final Report

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<p>Sodium channels from rat brain have been studied at the single channel level in planar bilayer membranes and by using <math>^{22}\text{Na}</math> tracer flux and <math>^3\text{H}</math>-STX binding methods. The rate of ion movement through the open pore and chemical modification of a specific saxitoxin blocking site at the external mouth of the pore led to a rate theory model of sodium influx and calcium block of the channel. Modulation of the channels by saxitoxin, batrachotoxin and scorpion and sea anemone polypeptide toxins was investigated using single channel analysis. The modulation of sodium channel gating by external and internal divalent cations was evaluated, leading to a novel theory of voltage-dependent channel gating. Saxitoxin-sensitive and insensitive sodium channels were studied in cultures of rat brain glial cells (astrocytes). The expression of sodium channels with high affinity for saxitoxin occurs spontaneously in culture but can be prematurely initiated by growth hormones.</p>					
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## SUMMARY

Sodium channels from rat brain have been studied at the single channel level in planar phospholipid bilayer membranes and in cultured glial cells using  $^{22}\text{Na}$  tracer flux and  $^3\text{H}$ -saxitoxin binding methods. In the presence of the activating neurotoxin, batrachotoxin (BTX), the channels were selective for sodium over other cations and the probability of being open ( $P_o$ ) was increased by membrane depolarization. Saxitoxin (STX) and tetrodotoxin (TTX) blocked the open channels inducing long-lived blocked periods corresponding to the toxin-bound state. Hyperpolarizing membrane potentials favored STX and TTX block with the  $K_i$  for block increasing about e-fold for 40 mV change in potential. Voltage dependence of block by TTX (monovalent cation) and STX (divalent) was the same, leading to the conclusion that voltage dependence of block is due to a voltage-driven conformational change in the binding site structure. External divalent cations (e.g.,  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ ) also blocked the open channels in a voltage-dependent manner, presumably by competing with permeant cation (e.g.,  $\text{Na}^+$ ) for one or more common sites in the channel pore. The carboxyl-methylating reagent, trimethylloxonium (TMO), applied from the extracellular side, abolished sensitivity to toxins, reduced single channel conductance and virtually eliminated block by external  $\text{Ca}^{++}$ . We conclude that the TMO-sensitive, toxin binding site may be an essential step in the ion permeation process. A three-site, four-barrier rate theory model has been developed to account for ion movement through the channel under a variety of experimental conditions assuming that permeant and blocking cations interact with the toxin binding site as they enter the channel. Biochemical experiments indicated that BTX and VER-activated Na channels can be purified from detergent extracts of rat brain and reconstituted in phospholipid vesicles. The purified channels can then be incorporated into planar bilayers for characterization. With this procedure, the two types of VER-activated Na channels were separated and were revealed to have slightly different molecular weights (235,000 and 265,000) and different single channel conductances when reconstituted into planar lipid bilayers. The opening and closing (gating) of batrachotoxin-activated sodium channels can be modulated by external and internal divalent cations with external divalents causing a depolarizing shift in the open probability vs membrane potential relation and internal divalents causing a hyperpolarizing shift. Although some of these effects are due to interactions of the divalent cations with negatively charged phospholipids, the predominant effect is due to binding to one or more sites on the intracellular and extracellular sides of the channel protein. The selectivity for various divalent cations on both sides differs from that for open channel block and competition with saxitoxin binding indicating that neither the saxitoxin binding site nor the "selectivity filter" within the channel pore is likely to be involved in the effects of divalents on gating. External divalents preferentially slowed the rate of channel opening whereas internal divalents preferentially slowed the closing rate. This suggests that internal and external divalents are capable of alternately interacting with elements of the voltage-sensing machinery of the channel that are alternately exposed to the extracellular or intracellular spaces as the channel closes and opens. In cultured neonatal rat brain glial cells (astrocytes), high affinity saxitoxin binding sites and saxitoxin sensitive  $^{22}\text{Na}$  influx are virtually absent until day 8 when the density of saxitoxin binding sites and saxitoxin blockable flux begin to increase. This spontaneous change can be prematurely initiated and greatly accelerated by placing the cells in a defined culture medium without fetal calf serum but with various growth factors. One or more of these medium components may regulate the differential expression of saxitoxin-sensitive and saxitoxin-insensitive sodium channels in these cells.

## FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. NIH 85-23, Revised 1985).

Animals are maintained in the Central Animal Facility of the University of Maryland School of Medicine. Animals are housed, cared for, and used strictly in accordance with USDA regulations. The University of Maryland School of Medicine Central Animal Facility is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. The program of animal care is directed by a full-time, specialty trained, laboratory animal veterinarian. This institution has an Animal Welfare Assurance on file with the NIH Office for Protection from Research Risks (OPRR), Assurance Number A3200-01

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## EXPERIMENTAL RESULTS

### A. Specific Aims of Original Proposal.

The original specific aims (listed below) have not changed. During the first year, substantial progress was made on aims 1, 2, and 4. As a consequence of recent results and developments in the field, new experimental approaches to address these aims have been introduced (see section D). Progress was made during the second year on aims 4 and 5. In addition work was expanded on aim 1 in a new area related to the development of STX and TTX binding and block in brain glial cells in culture (section F). During the final year research concentrated on the mechanisms of voltage-dependent sodium channel gating and its modulation by divalent cations and neurotoxins (aim 5).

1. to determine the molecular basis of the voltage dependence of saxitoxin (STX) and tetrodotoxin (TTX) block of neuronal sodium channels.

2. to examine the effects of trimethyloxonium (TMO; a modifier of the negatively-charged toxin binding site) on ion permeation through the channels and on channel block by calcium and strontium.

3. to examine the effect of other carboxyl modifying reagents on ion permeation and block. Special attention will be paid to carbodiimides which render sodium channels insensitive to TTX.

4. to utilize the information in 1 - 3 above to derive a rate theory model for ion permeation through the channel.

5. to determine the rates of opening and closing of single sodium channels at varying membrane potentials and the effects of TMO treatment on these processes.

## B. Publications and Scientific Meetings.

### Publications.

- Worley, J.F., R.J. French, and B.K. Krueger. 1986. Trimethyloxonium modification of single batrachotoxin-activated sodium channels in planar bilayers. *J. Gen. Physiol.* 87: 327-349.
- Krueger, B.K., J.F. Worley III, and R.J. French. 1986. Block of sodium channels in planar lipid bilayers by calcium and guanidinium toxins. Are the mechanisms of voltage dependence the same? *Annals of the New York Academy of Sciences.* 479: 257-268.
- French, R.J., J.F. Worley, W.F. Wonderlin, and B.K. Krueger. 1986. Three sites of calcium block in single sodium channels? *Proceedings of the Eighth Annual Conference of the IEEE Engineering in Medicine and Biology Society.* 962-965.
- Cukierman, S., W.C. Zinkand, R.J. French and B.K. Krueger. 1988. Effects of membrane surface charge and calcium on the gating of rat brain sodium channels in planar bilayers. *Journal of General Physiology* 92: 431-447
- Yarowsky, P.J. and B.K. Krueger. 1989. Development of saxitoxin-sensitive and insensitive sodium channels in cultured neonatal rat astrocytes. *Journal of Neuroscience* 9:1055-1061.
- Krueger, B.K. 1989. Toward an understanding of structure and function of ion channels. *FASEB Journal* 3: 1906-1914.
- Corbett, A.M. and B.K. Krueger. 1988. Polypeptide neurotoxins modify gating and single channel conductance of veratridine-activated sodium channels in planar lipid bilayers. *Journal of Membrane Biology*, in press.
- Wonderlin, W.F., French, R.J. and N.J. Arispe. Recording and analysis of currents from single ion channels. in press.
- Worley, J.F., R.J. French, and B.K. Krueger. Effects of divalent cations and membrane surface charge on ion permeation through single sodium channels from rat brain incorporated into planar lipid bilayers. submitted to *J. Gen. Physiol.*
- Worley, J. F., W.F. Wonderlin, B. K. Krueger, and R. J. French. Ion permeation, divalent ion block and chemical modification of single sodium channels. Description by a 4-barrier, single occupancy, rate theory model. submitted to *J. Gen. Physiol.*
- Cukierman, S. and B.K. Krueger. Modulation of sodium channel gating by external divalent cations studied in planar lipid bilayers: Differential effects on opening and closing rates. submitted to *Journal of General Physiology.*
- Cukierman, S., F.C. Albuquerque, and B.K. Krueger. Modulation of sodium channel gating by internal divalent cations provides evidence for strings of negative intrachannel gating charges. submitted to *Biophys. J.*

O'Leary, M.E. and B.K. Krueger. Batrachotoxin and  $\alpha$ -scorpion toxin stabilize the open state of single voltage-gated sodium channels. submitted to Molecular Pharmacology.

Abstracts:

- Worley, J. F., B. K. Krueger, and R. J. French. 1986. Methylation of a superficial carboxyl group in sodium channels reduces block by calcium, and unit conductance as well as abolishing block by saxitoxin. *Biophys. J.* 49: 42a.
- Corbett, A. M., W. C. Zinkand, and B. K. Krueger. 1986. Saxitoxin (STX) and calcium interactions with purified sodium channels reconstituted in planar lipid bilayers. *Soc. Neuroscience Abst.* 12: 45.
- Corbett, A. C., W. C. Zinkand, and B. K. Krueger. 1987. Activation of single neuronal sodium channels by veratridine and polypeptide neurotoxin in planar lipid bilayers. *Biophys. J.* 51: 435a.
- Yarowsky, P. J., R. Johnson, W. C. Zinkand and B. K. Krueger. 1987. Development of sodium channels in rat cerebral astrocytes. 1987. *Federation Proceedings* 46: 504.
- Corbett, A. M. and B. K. Krueger. 1988. Purification and characterization of two sodium channels from rat brain with different alpha subunits and distinct functional properties. *Biophysical Journal* 53: 15a.
- O'Leary, M.E. and B.K. Krueger. 1988. Gating of batrachotoxin-activated Na channels is altered by scorpion venom in planar lipid bilayers. *Society for Neuroscience Abstracts* 14: 835.
- Yarowsky, P.J., D.S. Brougher and B.K. Krueger. 1988. Induction of STX-sensitive sodium channels in cultured astrocytes. *Society for Neuroscience Abstracts* 14: 836.
- Cukierman, S., W. C. Zinkand, R. J. French, and B. K. Krueger. 1988. Effects of calcium and lipid surface charge on sodium channel gating. *Biophysical Journal* 53: 536a.
- O'Leary, M.E. and B.K. Krueger. 1988. Gating of batrachotoxin-activated Na channels is altered by scorpion venom in planar lipid bilayers. *Soc. Neuroscience Abstracts* 14:835.
- Yarowsky, P.J., D.S. Brougher and B.K. Krueger. 1988. Induction of STX-sensitive sodium channels in cultured astrocytes. *Soc. Neuroscience Abstracts* 14:836.
- O'Leary, M.E. and B.K. Krueger. 1989. Isolation of polypeptide toxins from the scorpion *Leiurus quinquestriatus* that alter the gating of batrachotoxin-activated sodium channels in planar lipid bilayers. *Biophys. J.* 313a.



### Scientific Meetings.

B. Krueger presented an invited paper at the New York Academy of Sciences conference on "Tetrodotoxin, Saxitoxin, and the Molecular Biology of the Sodium Channel", 12/85.

B. Krueger presented a paper at the Biophysical Society meeting, San Francisco, CA, 2/86.

B. Krueger attended the Gordon Research conference on "Ion Channels in Muscle and other Excitable Membranes", 8/86.

R. J. French presented a paper at the Eighth Annual Conference of the IEEE/Engineering in Medicine and Biology Society, Fort Worth, TX, 11/86. (B. K. Krueger, co-author)

B. K. Krueger, W. C. Zinkand and A. M. Corbett presented a paper at the Biophysical Society meeting, New Orleans, LA, 2/87.

B. K. Krueger, W. C. Zinkand and P. J. Yarowsky presented a paper at the FABEB meeting, Washington, D.C., 3/87.

S. Cukierman, R. French, B. Krueger and W. Zinkand attended the 1988 Biophysical Society meeting in Phoenix, AZ, and presented a poster on the effects of lipid surface charge on sodium channel gating.

B. Krueger and D. Brougher were coauthors on a poster on development and modulation of saxitoxin-sensitive sodium channels in astrocytes at the Annual Meeting of the Society for Neuroscience, Toronto, Ontario, Canada, 11/88.

B. Krueger and M. O'Leary presented posters on work supported by this contract at the Society for Neuroscience Meeting in November, 1988, in Toronto, Canada.

M.E. O'Leary and B.K. Krueger presented a paper at the Biophysical Society Meeting, Cincinnati, OH, 2/89.

### C. Rate Theory Modeling.

Methods. All experiments described in this report have been carried out using membrane vesicles prepared from homogenates of rat brain that are enriched in  $^3\text{H}$ -STX binding sites (1). Sodium channels were incorporated into phosphatidylethanolamine-phosphatidylserine planar bilayers and studied in the presence of the activating toxin, batrachotoxin (BTX; 2,3). We have described ion movement through BTX-activated sodium channels by assuming that each permeant ion must traverse a series of energy barriers as it moves through the channel pore. Between each pair of adjacent barriers is an energy minimum or well, occupancy of which is energetically relatively favorable. In general, according to such a so-called "rate theory" model, highly permeant ions encounter low energy barriers and shallow wells, whereas ions that encounter one or more very high barriers are impermeant and those that enter very deep wells are minimally permeant and actually block the flow of permeant ions. Voltage-dependent sodium channels have been modeled in this way (4) using a three-well (site), four-barrier rate theory model, however, the number of barriers and sites and their positions were chosen arbitrarily to allow for good fits to macroscopic current-voltage data. The basic outline of our modeling and the application of the model to the experimental data were described in report AN-1-1987.

Our model (Figure 1) was chosen to account for the following

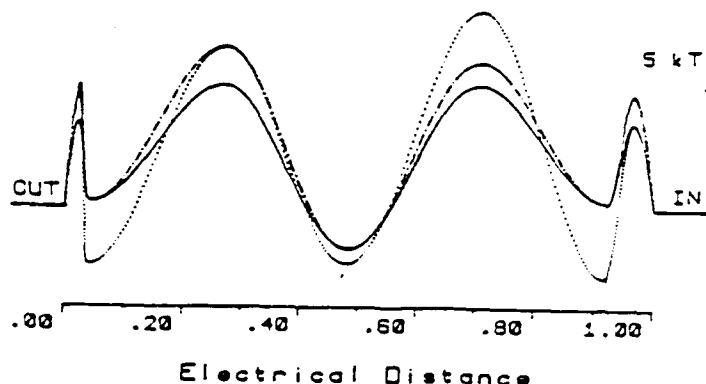


Figure 1. Energy profile for sodium (solid line), calcium (dotted line) and potassium (dashed line). In the rate theory model used in these studies, ions must traverse all four barriers to cross (permeate) the membrane through the channel.

experimental observations:

1. Trimethyloxonium (TMO) abolishes STX block and binding and profoundly affects sodium permeation and block by external calcium (5). Moreover, the STX binding site is probably located near the external surface of the channel (6). Thus, the saxitoxin binding site is assumed to be an initial binding site for entering cations and is represented by the external energy well.

2. External calcium blocks in a voltage-dependent manner with hyperpolarizing potentials favoring block (7). This is built into the model by placing a central site for either sodium or calcium reasonably deep within the channel so that entering cations experience a significant portion of the transmembrane electric field as they approach the site. Calcium blocks because it cannot readily traverse the high barrier just to the inside of this site.

3. Internal calcium blocks sodium ion movement through the channels in a slightly voltage-dependent manner suggesting a third site, accessible from and quite close to the intracellular side of the channel (Worley, Wonderlin, Krueger and French, submitted).

The flux equations were solved (by computer) for each ion, to generate predicted single channel current-voltage relations in the absence and presence of calcium (Figure 2) and single channel conductance-sodium concentration

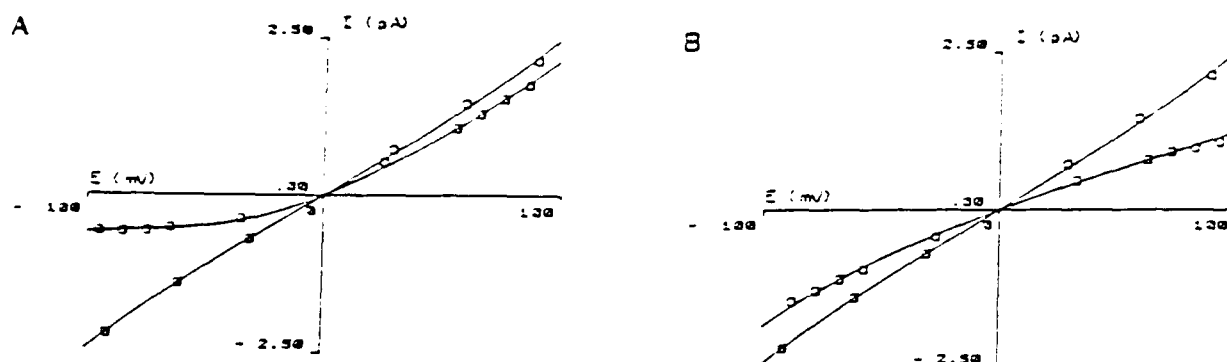


Figure 2. Single channel current vs. voltage relations for symmetrical 125 mM NaCl and 10 mM external (A) or internal (B) calcium. Lines are predictions from the rate theory model.

relations (Figure 3). Model parameters (energy levels) were varied to obtain a single set (Figure 1) that allowed satisfactory fits to all available data

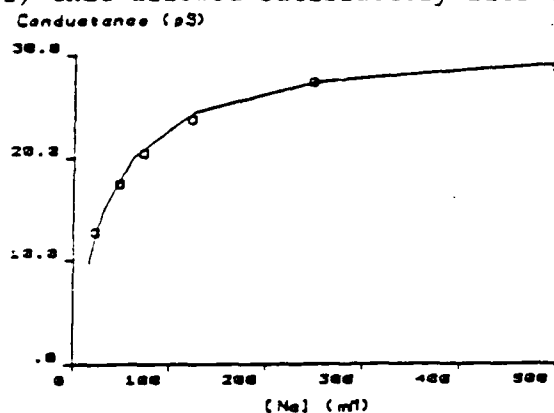


Figure 3. Single channel conductance vs. sodium concentration (symmetrical). The smooth line is the prediction from the rate theory model described above.

(Worley, Wonderlin, Krueger and French, submitted) including current-voltage relations and calcium block with "physiological" transmembrane ion concentration gradients (Figure 4). A critical test of this model was to mimic the effects of TMO on both sodium permeation and calcium block by manipulating only the exter-

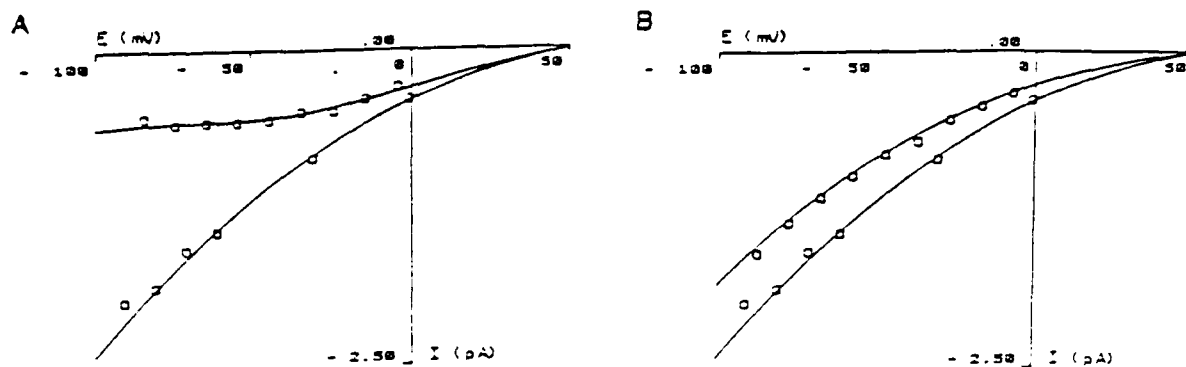


Figure 4. Single channel current vs. voltage relations for "physiological" ion gradients (125 mM Na/5 mM K outside; 125 mM K/5 mM Na inside) and 10 mM external (A) or internal (B) calcium. Lines are predictions from the rate theory model.

nal barrier and well. As shown in Figure 5, by making the outer barrier slightly higher and the outer well shallower for sodium and calcium, both the reduction in sodium conductance and the drastic decrease in potency of calcium block are

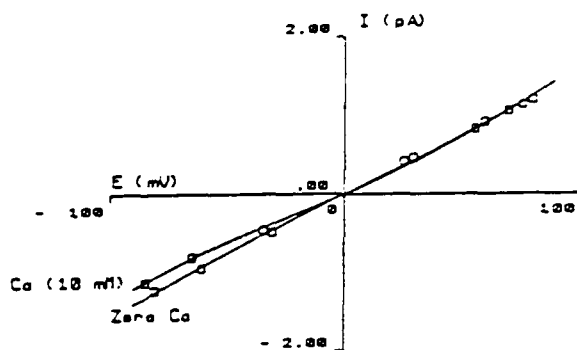


Figure 5. Single channel current vs. voltage relation for TMO-modified sodium channels in 125 mM symmetrical NaCl in the absence or presence of 10 mM external calcium. Compare with Figure 2A for unmodified channels.

accurately reproduced. It was concluded from these rate theory modelling studies that the experimental data are consistent with the saxitoxin binding site serving as a first and possibly essential step in the permeation of sodium through open sodium channels in the absence of blocking toxins.

Subsequent analysis revealed additional features. First using the optimal four-barrier, three-site model parameters, the occupancy by blocking calcium ion of each site was computed as a function of the membrane potential. The fraction blocked is proportional to the total occupancy. As can be seen from figure 1A below, where the occupancy of each site by calcium is indicated by the black vertical bar, external calcium can only enter the outermost two sites because the third (from the outside) barrier is too high to allow calcium to cross and permeate the channel. Hyperpolarization (more negative potentials) dramatically increases the channel occupancy (primarily at the central site) resulting in voltage-dependent block. In contrast, internal calcium (Figure 1B) can enter only the innermost site. Occupancy of this site is slightly favored by depolarization and thus block by internal calcium is only slightly voltage dependent.

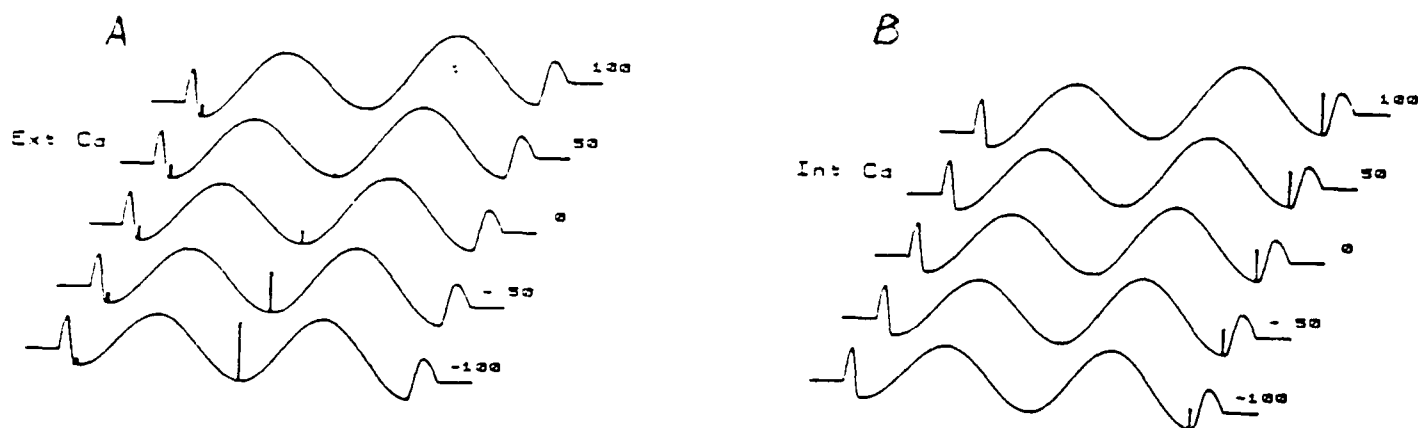


Figure 6. Calcium-occupancy in four-barrier, three-site model of the sodium channel in the presence of 125 mM  $\text{Na}^+$ . A. Probability of occupancy by  $\text{Ca}^{2+}$  vs  $V_m$  for 10 mM external  $\text{Ca}^{2+}$ . B. Same as A but internal  $\text{Ca}^{2+}$ .

These model predictions are summarized in Figure 7, where the total occupancy is plotted as a function of potential. Thus, at 10 mM external  $\text{Ca}^{2+}$ , the channel is much more occupied (blocked) at hyperpolarized potentials whereas at 10 mM internal calcium, the channel is nearly equally occupied (blocked) at all potentials. The fits of the model to the data are shown for external (Figure 8A) and internal (Figure 8B) calcium.

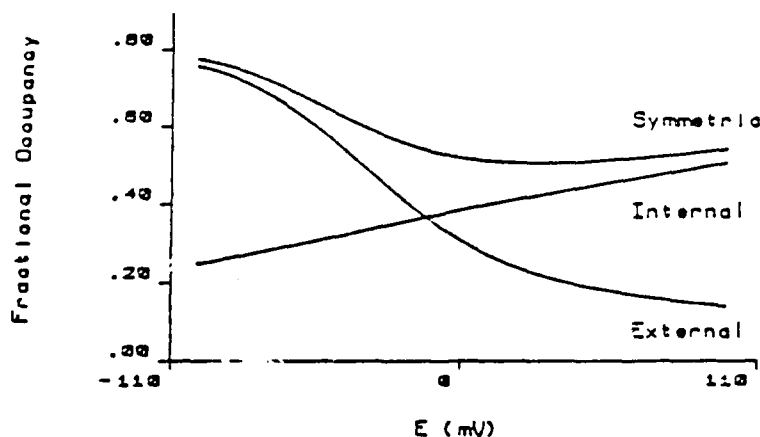


Figure 7. Total occupancy (related to block) by  $\text{Ca}^{2+}$  for rate theory model. Curves are shown for internal, external, and symmetrical, 10 mM  $\text{Ca}^{2+}$ .

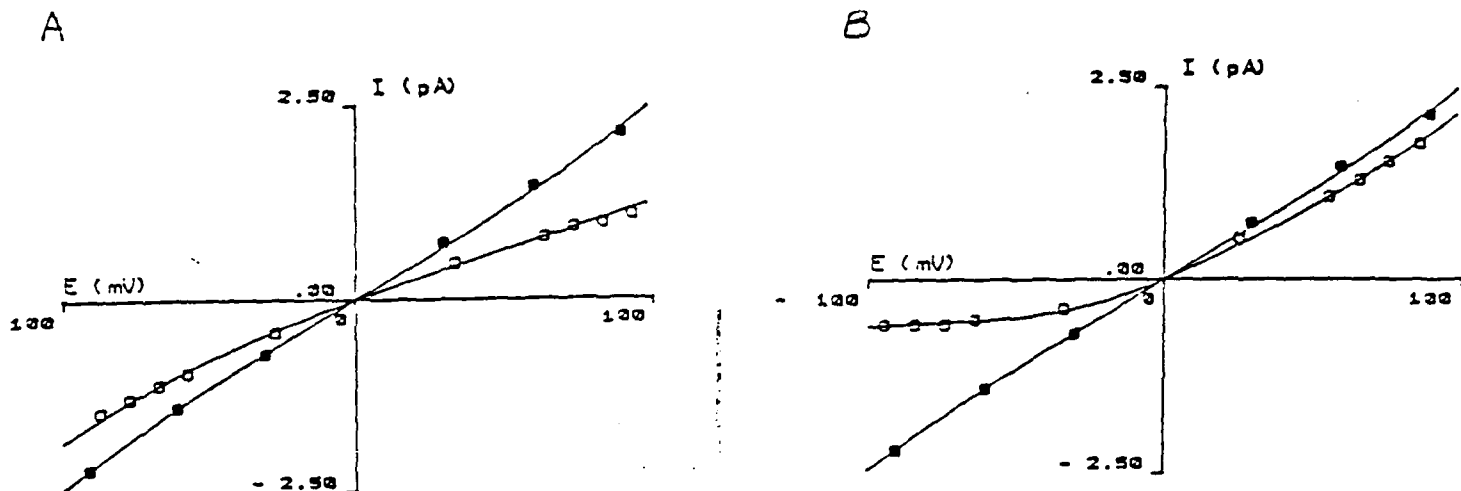


Figure 8. Fits of the model for symmetrical  $\text{Na}^+$  and internal (A) or external (B)  $10 \text{ mM Ca}^{2+}$ .

A second prediction of the model was that potassium ions should cause voltage-dependent block of the channels at depolarized potentials. The model prediction is shown in Figure 9 for symmetrical  $57 \text{ mM}$  sodium with and without symmetrical  $29 \text{ mM}$  potassium. It can be seen that only outward currents are blocked by potassium. This voltage-dependent block has recently been experimentally verified independently by Garber and Miller (8) on muscle sodium channels in planar lipid bilayers.

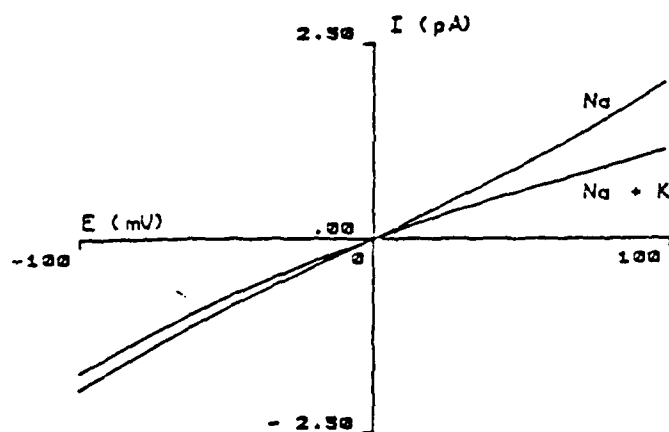


Figure 9. Rate theory model prediction of voltage-dependent block by symmetrical  $\text{K}^+$ .

D. The relationship between the saxitoxin binding site and the channel mouth.

It is usually assumed that saxitoxin and tetrodotoxin block sodium channels by binding in or near the external mouth of the channel pore and sterically occluding entry of permeant ions (9). Indeed, it is implicit in the rate theory modelling described above that block by STX may be due to competition between permeant ions and toxin for an essential binding site in the permeation pathway (Worley, Wonderlin, Krueger and French, submitted). With either of these mechanisms, toxin block may be said to be by "occlusion" on the channel. Some experimental evidence leads to questioning this idea and the proposal that STX and TTX might bind at a site distant from the mouth of the channel pore and block by an "allosteric" mechanism involving a conformational change in the channel protein structure (10). The allosteric mechanism has been suggested because the presence of a negatively charged (TMO-modifiable) carboxyl group associated with the toxin binding site and located at the mouth of the channel pore would distort the shape of the conductance-sodium concentration relation (11). The conductance-sodium relation is well described by a simple, rectangular hyperbola (12 and Figure 3) suggesting that there is little negative charge at the channel entrance. An additional test of the binding site-channel mouth relationship is to examine the effects of external divalent cations (e.g., calcium) on the voltage dependence of STX block. The voltage-dependence of block by guanidinium toxins is thought to be due to a voltage-dependent conformational change in the structure of the toxin binding site rather than to the toxins entering the membrane electric field because both STX (a divalent cation) and TTX (monovalent) show the same voltage dependence (6,10,12,13). Rando and Strichartz (14) have suggested that this voltage-dependent block occurs only when the channels are open. In contrast to voltage-dependent guanidinium toxin block, calcium blocks open sodium channels with a similar voltage dependence because it binds to a site deep within the channel (see section C above). In light of this information, external calcium should reduce the voltage dependence of toxin block (via an electrostatic interaction between calcium and toxins, both cations, in their respective binding sites) if the toxin binding site is close to the calcium binding site inside the channel pore (e.g., at the entrance to the channel). Figure 10 shows that external calcium substantially reduces the vol-

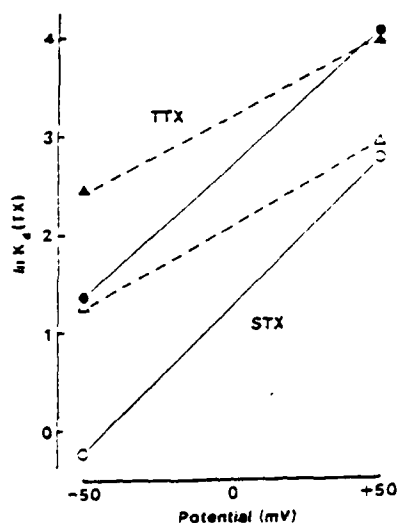


Figure 10. Semilog plot of  $K_i$  for STX and TTX block vs. membrane potential in the absence (solid lines) and presence (dashed lines) of 10 mM extracellular calcium.

tage dependence of STX and TTX block, a result consistent with a toxin binding site at the extracellular mouth of the pore. Green et al. (10) have reported that external zinc, which exhibits similar voltage-dependent block of sodium channels, does not affect the voltage dependence of guanidinium toxin block. At the present time, the spatial relationship between the STX binding site and the channel entrance is unclear and there is not sufficient information to unequivocally distinguish between the "occlusion" and "allosteric" models for toxin block.

#### E. Purification and Characterization of Two Sodium Channels.

<sup>3</sup>H-STX binding sites have been purified from homogenates of rat brain by solubilization in Triton X-100 and fractionation by normal and HPLC column chromatography (15). The binding sites were first fractionated by ion exchange and lectin-affinity chromatography. Concentrated samples were then sized by HPLC gel filtration and the final purification step was by HPLC DEAE ion exchange with a 100 - 250 mM KCl gradient. The overall purification scheme is summarized below:

#### SODIUM CHANNEL PURIFICATION

5-Step Method	Specific Activity (pmol/ml)	Yield (percent)	Purification (fold)
Solubilized P3	5.1	100	1
DEAE Sepharose	73	85	14
Hydroxylapatite	83	21	16
WGA	423	13	83
HPLC Molecular Sieve	1236	4.5	242
HPLC DEAE Gradient	2921	3.4	547

The final fractions are nearly pure (estimated specific activity of pure channels of molecular weight 315,000 is 3170 pmoles/mg). The purified <sup>3</sup>H-STX binding sites were reconstituted in phospholipid vesicles by removal of detergent and were incorporated into planar bilayers for determination of functional activity (2,16). In the presence of BTX, the channels were similar to unpurified channels incorporated directly from native membrane vesicles. Depolarizing potentials induced channel opening, the single channel conductance was about 25 pS in 150 mM NaCl and external STX blocked in a voltage-dependent manner (Figure 11).



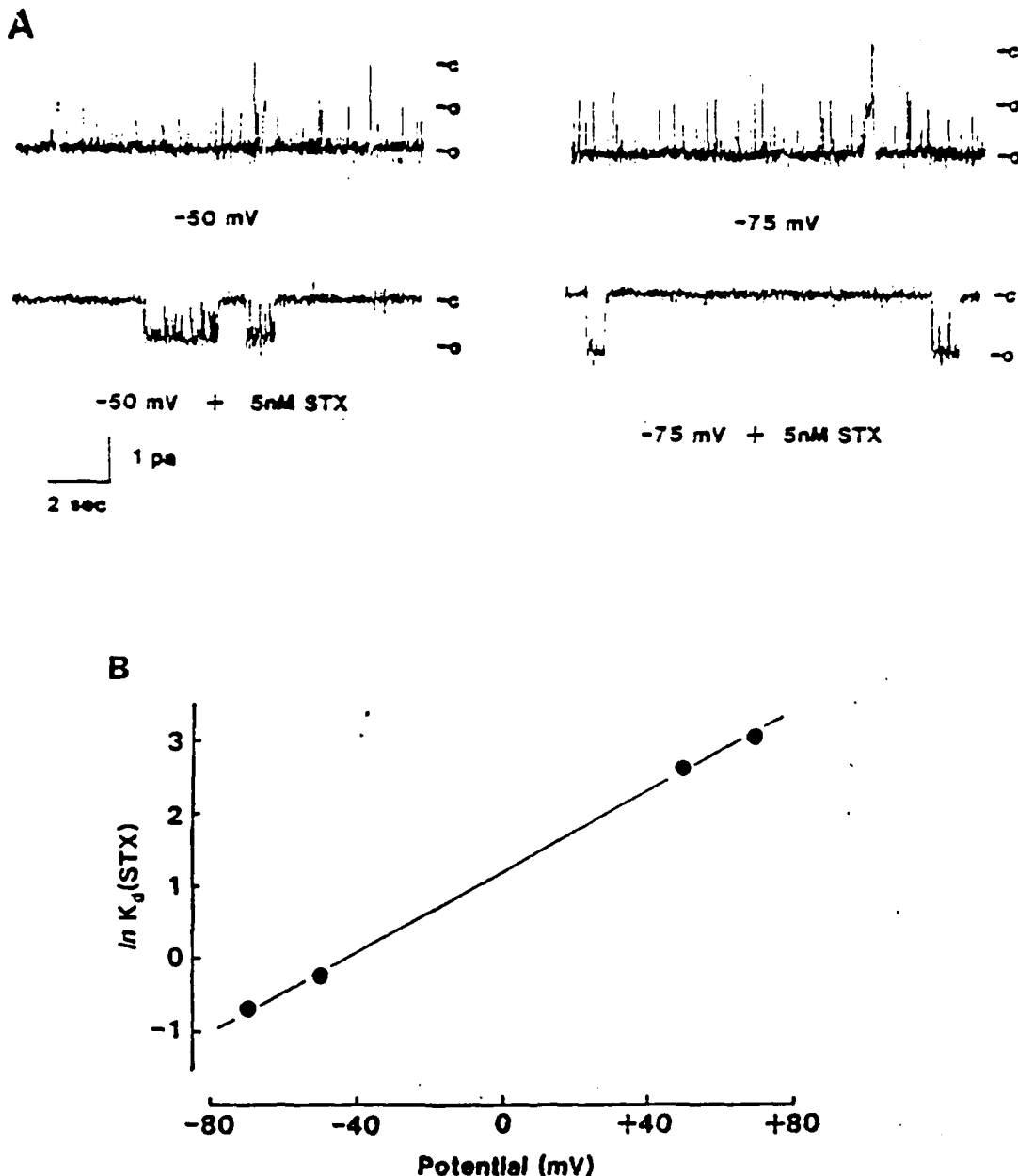


Figure 11. Voltage dependent block by STX. A. Two purified BTX-activated channels were incorporated into a planar bilayer. Five nM STX induced long-lived closing (blocked) periods. B. The potency of block (inversely related to  $K_d$ ) was greater at depolarized (positive) potentials.

In tracer flux experiments, veratridine (VER) has been found to act on sodium channels synergistically with polypeptide toxins from scorpion and sea anemone venoms (17). This synergism may also be seen in bilayer experiments (Corbett and Krueger, submitted and report AN-1-1986) in which polypeptide toxins increased the probability of the channel being open.

With purified channels two STX-sensitive conductance states were observed (about 15 and 25 pS in BTX and 5 pS and 10 pS in veratridine). Interestingly, sea anemone polypeptide neurotoxin dramatically increased the probability of being open for only the channels with the larger conductance (Figure 6). In

preliminary experiments, critical examination of the polypeptide composition of active  $^3\text{H}$ -STX binding fractions from the HPLC DEAE ion exchange column revealed that the early part of the peak was composed primarily of a 235 kD subunit whereas the later part of the activity peak contained a 265 kD subunit. Scorpion polypeptide toxin greatly increased the open probability of the large conductance channel (235 kD, 10 pS) and increased the single channel conductance of the 265 kD channel from 5 to 9 pS (Figure 12). The possible relationship of these two types of brain sodium channels to the two distinct sodium channel genes reported by Noda et al. (18) requires further investigation.

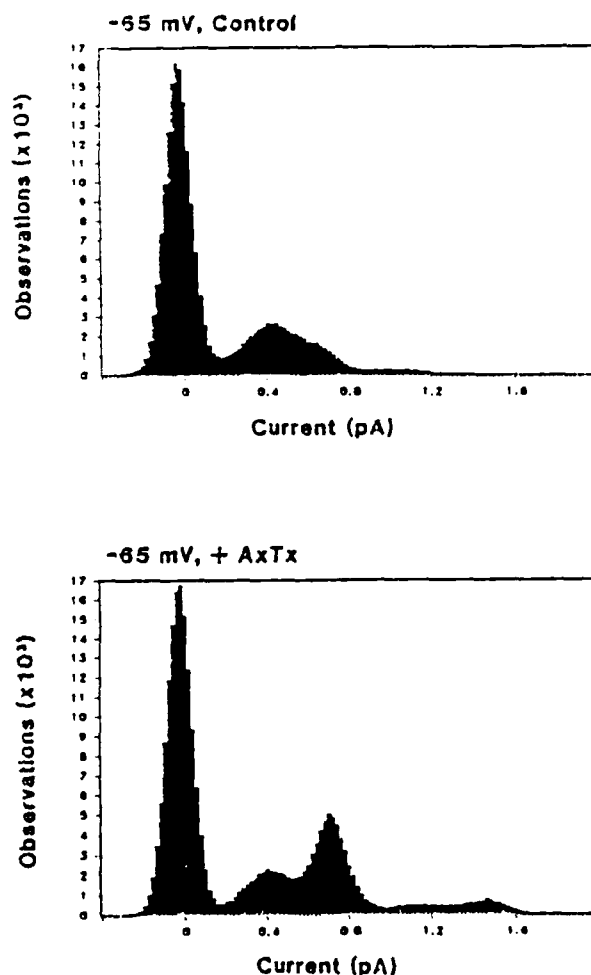


Figure 12. Amplitude histograms of purified, reconstituted sodium channels in planar bilayers in the presence of VER and sea anemone polypeptide toxin. Top: The large peak represents the closed state and the smaller peak is the combination of 5 and 10 pS sodium channels. Bottom: addition of 200 nM sea anemone toxin (AxTx) selectively increases the probability that the larger, 10 pS channel will be open.

#### F. Development and Modulation of STX/TTX-sensitivity of Sodium Channels in Rat Cortical Astrocytes.

In collaboration with Dr. Paul J. Yarowsky, Department of Pharmacology and Experimental Therapeutics, University of Maryland, we have been studying changes in the STX and TTX sensitivity of sodium channels in neonatal rat cortical astrocytes (19). These glial cells, previously thought to have few if any vol-

tage-dependent channels, have recently been shown to have a variety of channel types similar to those in nerve and muscle cells (20). In early cultures (up to about 7 days), there is substantial BTX-activated  $^{22}\text{Na}$ -influx but virtually no detectable high affinity  $^3\text{H}$ -STX binding activity. Block of influx by STX behaved as a single site blocking reaction with a  $K_i$  of more than 100 nM. From day 7 to 12, high-affinity ( $K_d = 1$  nM)  $^3\text{H}$ -STX binding activity increased rapidly (Figure 13) with no overall change in BTX-activated influx. At two weeks or later, there were two components of block, one with a  $K_i$  estimated to be somewhat less than 1 nM and the other with a  $K_i$  of 100 - 500 nM (Figure 14). We interpret these results to indicate that initially, the astrocytes have only STX/TTX-insensitive sodium channels and that from 7 - 12 days in culture, some of these are replaced with (possibly newly synthesized) STX-sensitive channels that may be similar to those in neurons with a  $K_d$  of about 1 nM.

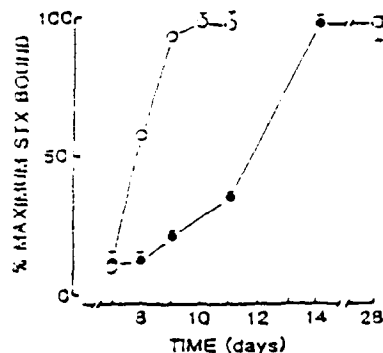


Figure 13. High affinity  $^3\text{H}$ -STX binding to neonatal rat brain astrocytes. The density of STX binding sites spontaneously increases between day 8 and day 12. (●), control; (○), chemically defined medium added on day 7.

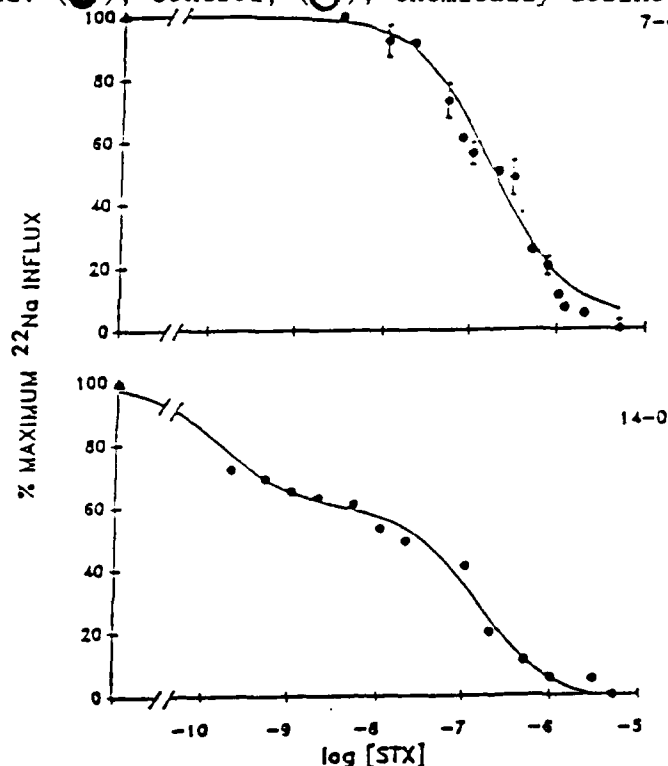


Figure 14. Two components of neurotoxin-stimulated  $^{22}\text{Na}$  influx into neonatal rat brain astrocytes. Top (7 day) : All influx is blocked by STX with a single  $K_i$  of about 100 nM. Bottom (14 day): About 40% of the influx is blocked by about 0.5 nM STX and the remainder by 100-200 nM STX.

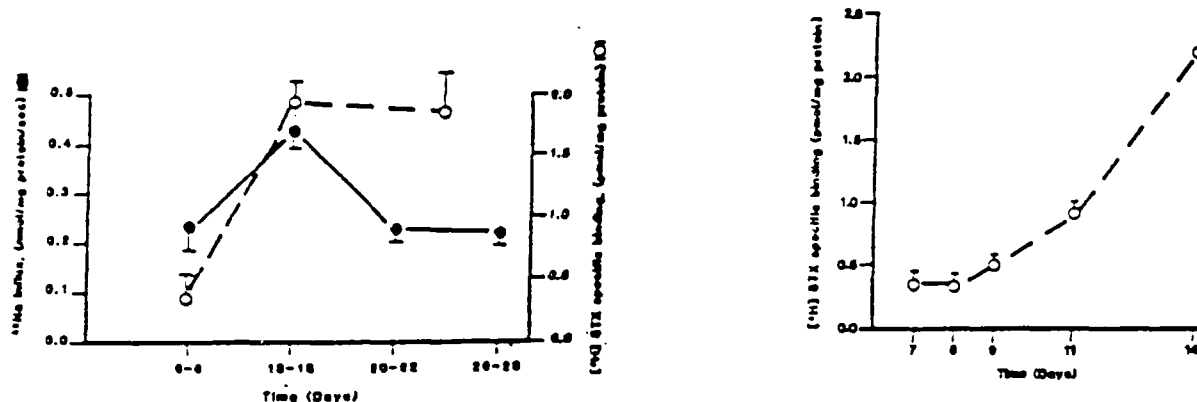


Figure 15. Changes in BTX-activated  $^{22}\text{Na}$  influx (filled circles) and  $^3\text{H}$ -STX binding (open circles) as a function of time in neonatal rat cortical astrocytes. Left: STX binding undergoes a five-fold change from day 6-8 to times older than 13 day. Sodium influx is about the same at early and later times. Right: detail of the increase in high affinity  $^3\text{H}$ -STX binding from day 7 to 14.

The appearance of high affinity  $^3\text{H}$ -STX binding sites developed over a six-day period from days 8 to 14 (Figure 15). During this time  $^{22}\text{Na}$  influx through Na channel was quite significant: up to one week in culture this flux was entirely through STX-insensitive channels (Figure 14, top). Concurrently with the appearance of high affinity STX binding sites (Figure 15), a component of influx sensitive to nanomolar concentrations of STX appears (Figure 14, bottom). This appearance of Na channels with high sensitivity to STX can be stimulated, within two days, by removal of fetal calf serum from the culture medium and simultaneous addition of chemically defined medium containing salts, amino acids and several polypeptide growth factors (19). We believe that one or more of these factors stimulates the appearance of high affinity channels.

#### G. Modulation of Sodium Channel Gating by Divalent Cations.

We have been studying the effects of external and internal divalent cations on the opening and closing (gating) of BTX-activated sodium channels in planar bilayers (21-23). It has been known for about 20 years that increasing external  $\text{Ca}^{2+}$  depresses excitability primarily by reducing the probability of opening of sodium channels (24). It has been unclear whether this effect is due to interaction of the divalent cations with negatively charged phospholipids or the channel itself, or both. We have addressed this problem by studying the effects of divalent cations on sodium channel gating in uncharged (phosphatidylethanolamine) or negatively charged (phosphatidylserine) bilayers. As shown in Figure 16, external  $\text{Ca}^{2+}$  causes a depolarizing shift in the probability of being open ( $P_o$ ) vs  $V_m$  in both neutral (left) and negatively charged (right) bilayers. The

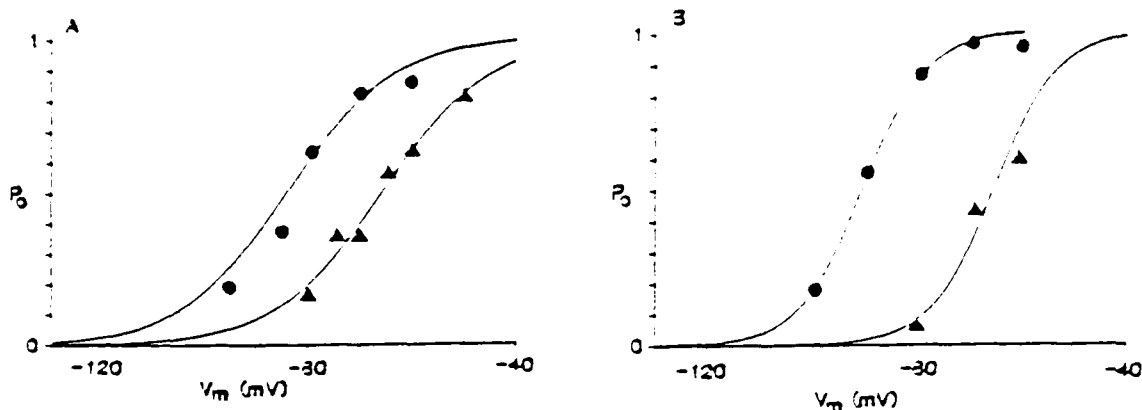


Figure 16.  $P_o$  vs  $V_m$  for a single sodium channel in a neutral (PE) bilayer (A) and in a negatively charged (PS) bilayer (B). Methods are described in reference 1. (●), control; (▲), + 10 mM  $Ca^{2+}_o$ .

shift in negative bilayers was always larger. Thus, some of the effects of divalent cations are due to an interaction with the protein, although addition of negatively charged lipids also causes a small amount of hyperpolarizing shift. Complementary results were obtained with internal  $Ca^{2+}$ , viz., a hyperpolarizing shift was observed that was larger in negative bilayers. This is summarized in Figure 17. In neutral bilayers (A), external  $Ca^{2+}$  caused a

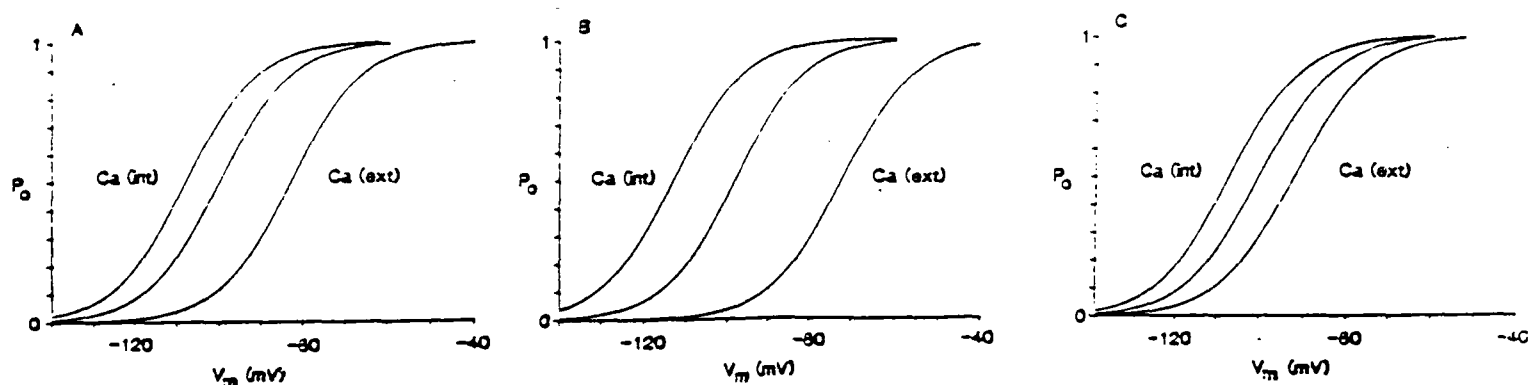


Figure 17.  $Ca^{2+}$ -induced shifts in sodium channel gating. Curves show the average shift in  $P_o$  vs  $V_m$  relations for external or internal  $Ca^{2+}$  (10 mM). A. results in neutral bilayers; B, negatively charged bilayers; C. Effect attributable to negatively charged lipids, i.e., shift in negative lipids minus shift in neutral lipids.

depolarizing shift whereas internal  $Ca^{2+}$  caused a hyperpolarizing shift. Both effects were larger in negative bilayers (B). In both bilayers, the effect of external  $Ca^{2+}$  was larger than that of internal  $Ca^{2+}$  possibly due to a higher density of binding sites on the outside of the channel. The net shifts due to the negative lipids (C: B-A) were symmetrical (i.e., the same for internal and

external  $\text{Ca}^{2+}$ ) indicating that the influence of the negatively charged lipid headgroups was of equal strength from both the external and internal sides of the bilayer.

Since the STX binding site at the external mouth of the channel pore is negatively charged (5), it was of interest to determine if  $\text{Ca}^{2+}$  binding at this site accounted for some of the observed effects on gating. To address this question, we compared the shifts in the midpoint of the Na channel  $P_o$  vs  $V_m$  relations with the degree of block of the channel, for each of several divalent cations. As shown in Table I, the order of efficacy for shifting was similar but not identical to that for block. Thus, it is unlikely that the STX binding site is involved in the modulation of channel gating by divalent cations.

Table IA. Divalent Cation-Induced Shifts in  $V_{0.5}$

	$\Delta V_{0.5}$	
	NEUTRAL	NEGATIVELY CHARGED
0.5 mM $\text{Zn}^{2+}$	$10.2 \pm 1.3$ mV (3)	$14.4 \pm 1.7$ mV (3) *
5.0 mM $\text{Ca}^{2+}$	$16.3 \pm 1.0$ mV (4)	$21.6 \pm 3.0$ mV (4) *
5.0 mM $\text{Ba}^{2+}$	$13.6 \pm 0.8$ mV (5)	$18.1 \pm 1.1$ mV (5) **
5.0 mM $\text{Sr}^{2+}$	$8.7 \pm 1.2$ mV (4)	$16.9 \pm 2.3$ mV (5) #
5.0 mM $\text{Mg}^{2+}$	$8.5 \pm 2.0$ mV (3)	$15.0 \pm 0.5$ mV (3) ###

$\Delta V_{0.5}$  is the depolarizing shift in the midpoint of the  $P_o$  versus  $V_m$  relation induced by external divalent cations. Neutral bilayers were composed of 80% PE, 20% PC. Negatively-charged bilayers were composed of 56% PE, 44% PS. The data show means  $\pm$  s.e.m (number of observations). For each divalent cation,  $\Delta V_{0.5}$  was significantly larger in negatively charged bilayers (\*,  $p < .05$ ; \*\*,  $p < .005$ ; #,  $p < .07$ ; ###,  $p < .02$ ). Methods are described in ref. 1.

Table IB. Block of Na Channels by External Divalent Cations

	NEUTRAL	NEGATIVELY CHARGED
5.0 mM $\text{Zn}^{2+}$	$77 \pm 1\%$ (3)	77% (2) *
0.5 mM $\text{Zn}^{2+}$	$39 \pm 1\%$ (4)	$36 \pm 1\%$ (4) #
5.0 mM $\text{Ca}^{2+}$	$46 \pm 1\%$ (3)	$50 \pm 1\%$ (4) #
5.0 mM $\text{Mg}^{2+}$	$42 \pm 1\%$ (5)	$39 \pm 2\%$ (5) #
5.0 mM $\text{Sr}^{2+}$	29% (2)	$31 \pm 3\%$ (4) #
5.0 mM $\text{Ba}^{2+}$	$16 \pm 2\%$ (5)	$13 \pm 1\%$ (6) #

The membrane potential was -80 mV. Neutral bilayers were composed of 80% PE, 20% PC; negatively-charged bilayer were composed of 56% PE, 44% PS. Block is expressed as  $i_{\text{Me}^{2+}}/i_{\text{max}} \times 100\%$ . The data show means  $\pm$  s.e.m (number of observations). For each divalent cation, block was no greater in negatively charged bilayers than in neutral bilayers (\*,  $p > 0.98$ ; #,  $p > .99$ ).

We have also studied the effects of external and internal divalent cations on the rates of channel opening and closing (22,23). For this purpose, we used  $\text{Ba}^{2+}$  which is a good shifter (Table IA) but a poor blocker (Table IB). This problem was addressed by determining the single channel open and closed dwell time distributions which were well described by single exponentials at the potentials where gating is most voltage-dependent. Under these conditions, the

mean closed time is the reciprocal of the opening rate and the mean open time is the reciprocal of the closing rate. As shown in Figure 18A, external  $Ba^{2+}$  both increased the closing rate and decreased the opening rate, but had a larger effect of the opening rate. Internal  $Ba^{2+}$  decreased the closing rate and increased the opening rate but had a larger effect of the closing rate (Figure 18B). In 14 experiments, external  $Ba^{2+}$  doubled the closing rate and tripled the opening rate. In 12 experiments, internal  $Ba^{2+}$  tripled the closing rate and only doubled the opening rate.

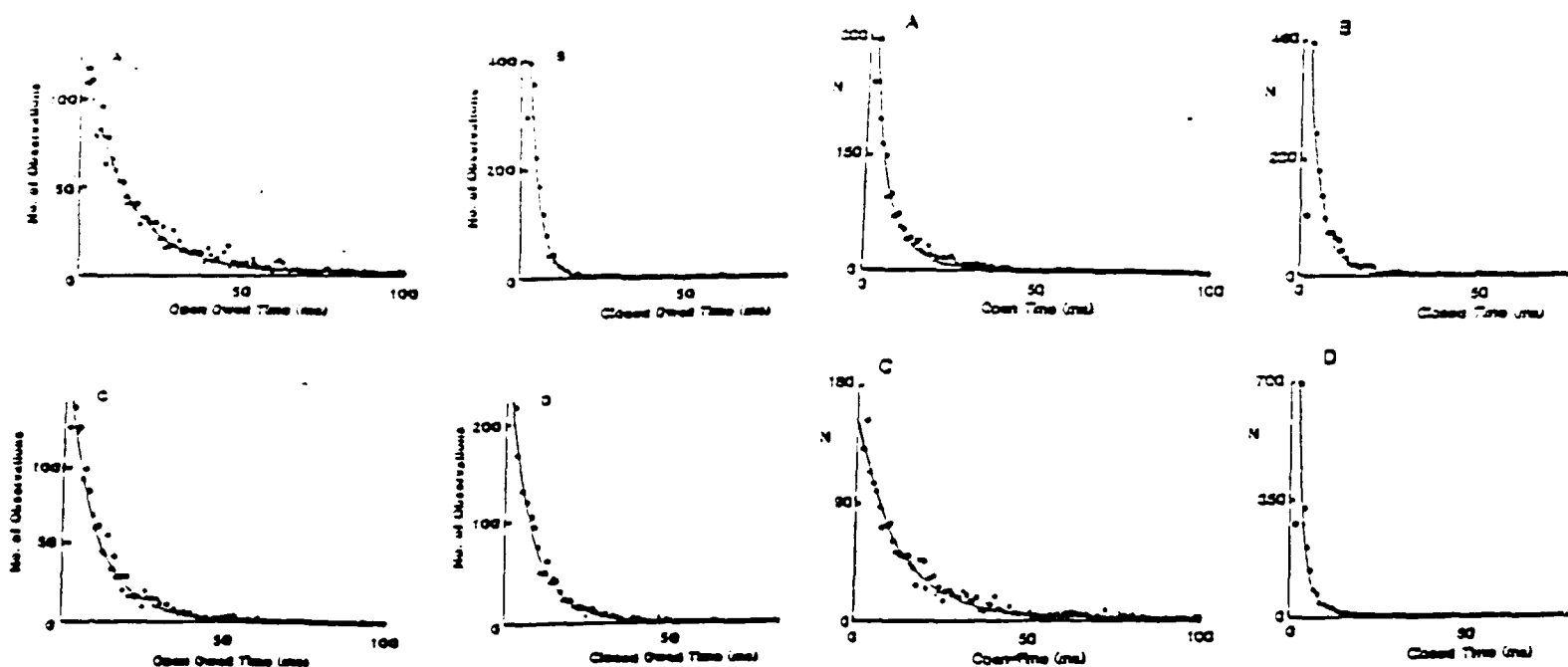


Figure 18. Distributions of open (A and C) and closed (B and D) dwell times of a single Na channel in the absence of divalent cations (A, B) and after addition of 5 mM  $Ba^{2+}$  (C, D) to the extracellular side (left) or the intracellular side (right). Membrane potential = -84 mV (l) and -98 mV (r). The graphs are plots of the number of openings (N) of duration t versus duration. Opening and closing rate constants were computed as the reciprocals of  $s_c$  and  $s_o$ , respectively. Methods are described in refs. 22 and 23.

We interpret our data to suggest that in the closed state a negatively charge component of the channel's gating apparatus is exposed to the outside where it can bind a divalent cation. The charged group moves into the channel protein as the channel opens but can only do so when the divalent cation dissociates, thus accounting for the preferential slowing of the opening rate. The effect of internal divalent cation is complementary, causing preferential slowing of the opening rate. As shown in Figure 19, we believe that these charges are the terminal charges on strings of negative charges within the channel protein that move outward as the channel closes. Recent molecular biological studies have identified segments in the primary amino acid structure of the channel that might be candidates for these gating charges (18).

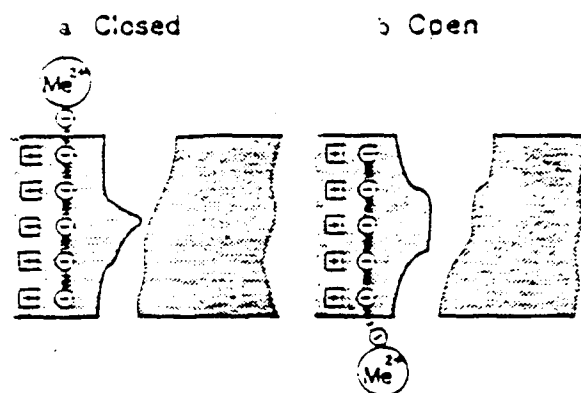


Figure 19. A hypothetical model for certain structural elements of the voltage-gated Na channel that is consistent with our results. Negative charges ( $\ominus$ ) may be aspartate or glutamate residues on a transmembrane helix, of which there are probably four. Countercharges ( $\oplus$ ) may be arginine residues on S4 (7). The terminal negative charge on the extracellular side of the channel is exposed and able to bind divalent cations only when the channel is closed (a). The internal terminal charge is exposed only when the channel is open (b); intracellular divalent cations can bind to this charge, immobilize the gating mechanism, and stabilize the open state.

#### H. Batrachotoxin+ $\alpha$ scorpion toxin-activated sodium channels do not close.

In the presence of batrachotoxin (BTX), Na channels display voltage-dependent gating with a midpoint of the open probability ( $P_o$ ) vs  $V_m$  relation being -90 to -100 mV (c.f., Fig. 16 and ref. 3, 21). Scorpion venom (*L. quinquestratus*) or a scorpion toxin, a polypeptide toxin purified from that venom, affects Na channel gating by slowing inactivation and by potentiating the activating effects of BTX. Scorpion toxin binds to the channels with high affinity ( $\approx 1$ -10 nM) at a site distinct from that of BTX or STX (25). We have found that addition of either scorpion venom or a scorpion toxin, in the presence of BTX holds the channels in an open configuration at potentials as negative as -140 mV (Fig. 20).



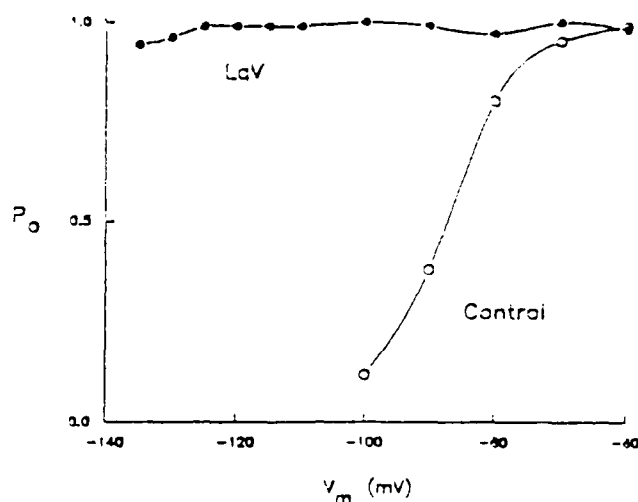


Figure 20. Probability of being open ( $P_o$ ) vs  $V_m$  for single BTX-activated Na channels in the absence or presence of scorpion venom (*L. quinquestriatus quinquestriatus*). The channel is open nearly all of the time at potentials up to -140 mV, the most negative potential normally tolerated by the planar bilayer. (○), control; (●), + 100 nM scorpion venom (LqV).

This capability to monitor single channel current at very large negative potentials provides the opportunity to study previously inaccessible aspects of ion permeation and block and the mechanisms of STX/TTX block. For example,  $\text{Ca}^{2+}$  is only slightly permeable through Na channels and detection of single channel currents carried by  $\text{Ca}^{2+}$  is not possible with present methods. At very negative potentials, some  $\text{Ca}^{2+}$  may be driven past the blocking site resulting in release of block which may be detectable. STX block has been demonstrated to be voltage-dependent with hyperpolarizing potentials favoring block (decreasing  $K_i$ ; refs. 3, 10, 13). The voltage-dependence of STX and TTX block is similar suggesting that the voltage dependence arises from a voltage-dependent conformational change in the toxin binding site rather than from entry of the toxin into the channel pore (6). This hypothesis could be directly tested by measuring block at very large negative potentials where a limiting high affinity state would be expected to be reached. The extended potential range available with the BTX + scorpion toxin-activated channels should provide this capability.

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